

## TECHNOLOGY REPORT

Generation of Mice With a Conditional Allele for *Trim33*Jieun Kim<sup>1,2</sup> and Vesa Kaartinen<sup>1,2\*</sup><sup>1</sup>Developmental Biology Program, The Saban Research Institute of Childrens Hospital Los Angeles, Departments of Pathology and Surgery, The Keck School of Medicine, University of Southern California, Los Angeles, California<sup>2</sup>University of Michigan, Department of Biologic and Materials Sciences, Ann Arbor, MI

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**Summary:** *Trim33* (*Tif1 $\gamma$* , *ectodermin*, *moonshine*), a member of the TIF1 family of transcriptional coactivators and corepressors, is a large nuclear protein that contains an N-terminal tripartite (Trim) domain composed of a RING domain, two B-box domains, and a coiled coil domain. It has been suggested that *Trim33* (*Ectodermin*) mediates ectodermal induction in the *Xenopus* by functioning as a *Smad4* ubiquitin ligase, while in the zebrafish *Trim33* (*moonshine*) has been reported to act as a R-Smad binding protein in induction of erythroid differentiation. Since the developmental role of *Trim33* in mammals is currently unknown, we generated mice carrying the conditional *Trim33* (*Trim33<sup>Fx</sup>*) allele by flanking exons 2–4 encoding most of the functionally critical N-terminal tripartite domain by loxP sites. We confirmed the null genotype by using the *Ella-Cre* transgenic approach to create mice that lack exons 2–4. Embryos deficient in *Trim33* die during early somitogenesis, demonstrating that *Trim33* plays an important nonredundant role in mammalian embryonic development. *genesis* 46:329–333, 2008. © 2008 Wiley-Liss, Inc.

**Key words:** *Trim33*; conditional knockout; *Tif1 $\gamma$* ; *ectodermin*; TGF- $\beta$

*Trim33* (*Tif1 $\gamma$* ) is one of 70 tripartite motif-containing Trim proteins (Yan *et al.*, 2004). Together with *Trim24* (*Tif1 $\alpha$* ) and *Trim28* (*Tif1 $\beta$* ), it forms a transcription intermediary factor-1 (*Tif-1*) subfamily of transcriptional regulators (Venturini *et al.*, 1999). Although *Tif-1* proteins all share several characteristic functional domains, for example, amino terminal Trim domains, carboxy terminal PHD finger, and Bromo domains, it is thought that their biological functions are quite divergent. For instance, *Trim28*, but neither *Trim24* nor *Trim33*, has been shown to interact with members of the KRAB zinc finger proteins (Friedman *et al.*, 1996; Kim *et al.*, 1996). Moreover, *Trim28* is an intrinsic component of the histone deacetylase N-CoR1/HDAC3 complex (Underhill *et al.*, 2000), whereas *Trim24* does not bind nuclear hormone receptors, but it has been shown to function as a coactivator of the retinoid acid receptor (Fraser *et al.*, 1998). Whereas mouse embryos deficient in *Trim28* die

soon after implantation, *Trim24* is not essential for embryogenesis, but was found to be a potent liver-specific tumor suppressor (Cammass *et al.*, 2000; Khetchooumian *et al.*, 2007). Much less is known about the biological role of *Trim33*, particularly in mammals. This is largely because of the fact that knockout mice have not yet been developed. However, it was recently shown that in *Xenopus* *Trim33* functions as an E3 ubiquitin-protein ligase promoting *Smad4* degradation via the ubiquitin proteasome pathway (Dupont *et al.*, 2005). In the zebrafish, *Trim33* was shown to be required for erythroid lineage-specific control of hematopoietic gene expression (Ransom *et al.*, 2004). Subsequently, it was shown that R-Smad/*Trim33* interaction is required for Tgf- $\beta$ -dependent erythroid differentiation. Interestingly, it seems that in this context *Trim33* does not target *Smad4* for degradation, but rather competes with it by binding activated R-Smads (He *et al.*, 2006).

To address the role of *Trim33* in mammalian embryogenesis, we generated mice harboring the conditional knockout allele for *Trim33*. Our strategy was to flank exons 2–4 encoding the functionally critical RING and B1 and B2 Box domains by loxP sites. The short (1.6 kb) and long (8.2 kb) arms of the targeting vector were PCR amplified from a Bac genomic DNA. The loxPNeoloxP cassette was inserted into intron 1 and a single loxP site was inserted into intron 4 (see Fig. 1). As a negative selection marker we used the *diphtheria toxin* (*DT*) gene. The linearized targeting vector was electroporated into TVB2 embryonic stem cells as described (Yang and Kaartinen, 2007). Fifteen out of 261 G418-resistant colonies were targeted to the correct locus; however, only three of them contained the 3' loxP site. All three correctly targeted ES clones were able to produce highly

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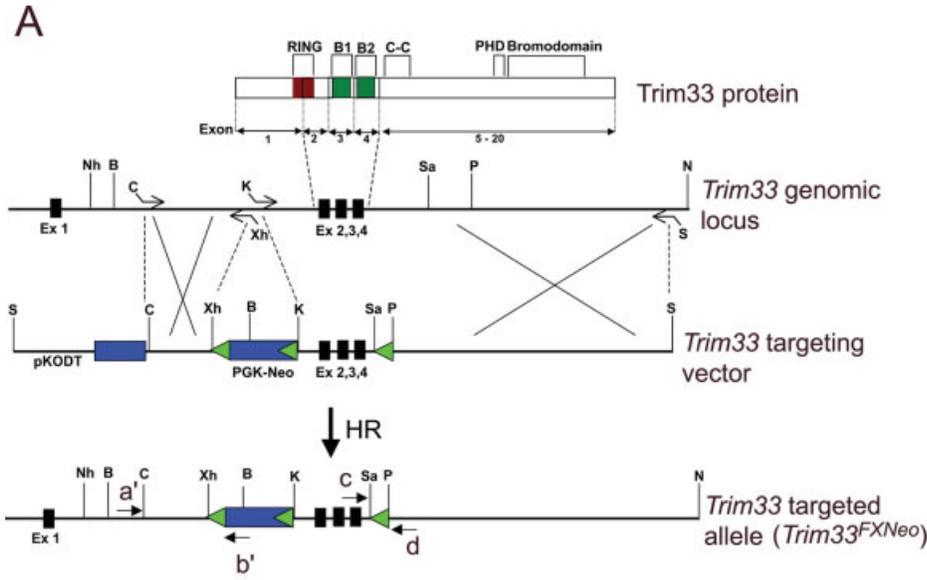
E-mail: vesak@umich.edu

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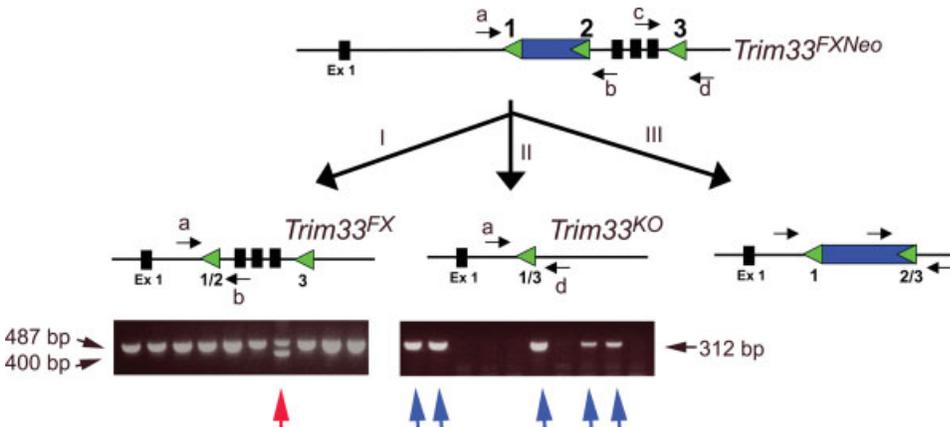
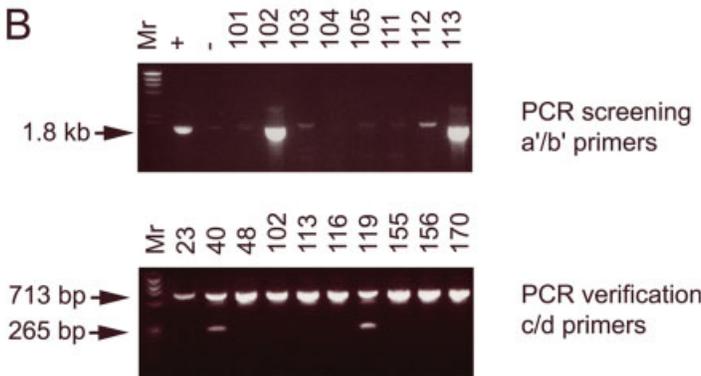
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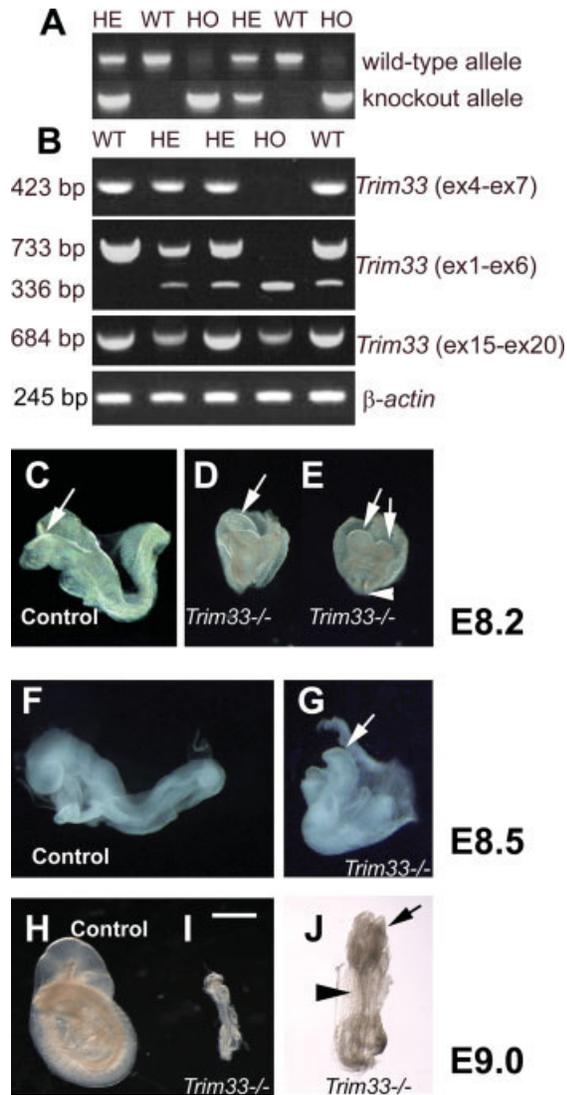
**FIG. 1.** *Trim33* targeting vector and screening of ES colonies. **A:** A schematic presentation of the Trim33 protein domains, *Trim33* genomic locus depicting a segment from exon1 to intron 4, the *Trim33* targeting vector and the *Trim33* targeted allele. **B:** An example of a PCR screen (upper panel); positive clones #102 and #113 can be easily identified. PCR analysis using c/d primers and template DNA from clones that suggested the correct targeting demonstrated that only clones number #40, #119, and #259 (not shown) had retained the 3' loxP site (lower panel). The wild type allele produces a 713-bp amplification product. Since the strategy to insert the 3' loxP site into intron 4 involved the replacement of a 553-bp SacI-PstI fragment with a loxP site, the mutant allele gives rise only to a 265-bp amplification product.



**FIG. 2.** Generation of mice carrying the floxed (*Trim33*<sup>FX</sup>) and knockout (*Trim33*<sup>KO</sup>) *Trim33* alleles. Transgenic *Ella-Cre* mice were crossed with mice homozygous for the targeting vector. The obtained mosaic males were further crossed with wild-type females to obtain *Trim33*<sup>FX/WT</sup> mice (type I recombination; red arrow) and *Trim33*<sup>KO/WT</sup> mice (type II recombination; blue arrows).

chimeric male mice, which in turn were potent germ line transmitters. To remove the *Neo* selection marker and to generate a presumed knockout allele for *Trim33*, we crossed the *Trim33*<sup>FXNeo/FXNeo</sup> mice (homozygotes for the targeting vector) with *Ella-Cre* transgenic mice

(Xu *et al.*, 2001). In these mice, *Cre* is expressed under the control of the adenoviral *Ella* promoter that targets expression of the Cre recombinase to the early mouse embryo (Lakso *et al.*, 1996). The *Ella-Cre* transgene creates both partial and complete recombinations and



**FIG. 3.** Development of embryos deficient in *Trim33* is arrested at the early somitogenic stage. **A:** PCR genotyping of embryos from the crossing between *Trim33*<sup>KO/WT</sup> males and females at E8.5. HE, heterozygotes; WT, wildtype; HO, homozygotes. **B:** RT-PCR analysis of embryos harvested at E8.5 using primers for exons 4 and 7 demonstrates that *Trim33*<sup>KO/KO</sup> embryos do not display any detectable mRNA product, while wild-type and heterozygote controls show an expected 423-bp PCR product (B, upper panel). Corresponding analysis using primers for exons 1 and 6 show that the wild-type sample produces the expected 733-bp PCR product, the homozygote mutant sample produces the expected 336-bp product, while the heterozygote samples give rise to both 733-bp and 336-bp products (second panel from the top). Corresponding analysis using primers for exons 15 and 20 shows that both the wild-type and mutant alleles produce a 684-bp amplification product (second panel from bottom).  $\beta$ -actin specific primers produced a 245-bp amplification product with comparable intensity from all samples (bottom panel). At E8.2–8.5, *Trim33* null mutants (**D, E, G**) demonstrate retarded development when compared to controls (**C, F**). **D** and **E** depict both lateral and frontal images of the same embryo, respectively. **I:** Development of the *Trim33*<sup>-/-</sup> embryos is arrested at the early somitogenic stage (E9.0). **J:** A high power picture of a *Trim33*<sup>-/-</sup> embryo shown in (**I**). **H:** Control littermate. Arrows in **C, D, E, G,** and **J** point to head folds, white arrowhead in **E** points to the primitive streak, and black arrowhead in **J** points to the somites.

**Table 1**  
Genotype Analysis of Offspring from *Trim33*<sup>+/-</sup> Intercross

Developmental stage	Somite pairs	<i>Trim33</i> <sup>-/-</sup>	<i>Trim33</i> <sup>+/-</sup>	Wild-type
Postnatal		0	12	2
E9.5		1	8	0
E9.0		2	4	4
E8.25–E8.5	5–6	6	4	2
E8.0	3	4	7	4

therefore *Ella-Cre* transgenic mice can be used both as a deleter mouse to generate knockout alleles and to remove selection markers (e.g., *loxP-pGKNeo-loxP*) when a triple loxP-strategy is used (Holzenberger *et al.*, 2000; Xu *et al.*, 2001). The obtained mosaic male mice progeny were subsequently crossed with wild-type female mice to obtain the floxed (*Trim33*<sup>FX</sup>) and null (*Trim33*<sup>KO</sup>) alleles (see Fig. 2). Homozygote *Trim33*<sup>FXFX</sup> mice were viable and fertile, and they did not display any recognizable phenotypes.

To confirm that the *Trim33*<sup>KO</sup> allele encoded the true null allele and to provide initial information about the biological role of *Trim33* during embryogenesis, we intercrossed the heterozygote *Trim33*<sup>KO/WT</sup> mice to obtain homozygote *Trim33*<sup>KO/KO</sup> mice (Fig 3A). Genotype analyses of newborn offspring revealed that all the homozygote mutant pups died during gestation (Table 1). To examine the time frame during which embryonic lethality occurred, we harvested embryos at different time points and discovered that at E9 the mutant embryos displayed a dramatic developmental delay when compared with controls (Table 1 and Fig. 3H–J). Nevertheless, they had formed a body axis, displayed head folds and the neural tube, and showed 5–6 somite pairs. We were unable to discover any living mutant embryos after E9.5. At E8.0–8.5 (3–6 somite pairs in controls), the *Trim33* mutant embryos were aligned at the base of the yolk sac, and while they had formed the anterior–posterior body axis and identifiable anterior structures, for example, head folds (arrows in Fig. 3D, E, and G), it was difficult to identify any other embryonal structures. RT-PCR using primers with target sequences in exons 4 and 7 did not produce any detectable amplification product, while the heterozygote and wild-type samples showed the expected 423-bp fragment (Fig. 3B). This is concordant with the lack of sequences encoded by exon 4. To further validate the *Trim33*<sup>KO/KO</sup> allele, we used exon 1- and exon 6-specific primers. The wild-type and homozygote *Trim33*<sup>KO/KO</sup> alleles produced the expected 733-bp and 366-bp products, respectively, while the heterozygote samples gave rise to both PCR fragments (Fig. 3B). RT-PCR analysis using primers specific for 3' exons 15 and 20 produced the expected 684-bp fragment from wildtype, heterozygote (*Trim33*<sup>KO/WT</sup>) and homozygote (*Trim33*<sup>KO/KO</sup>) samples suggesting that the mutated allele lacking exons 2–4 is able to produce a stable mRNA (Fig. 3B). To conclude, our RT-PCR analyses demonstrated that the mRNA encoded by *Trim33*<sup>KO/KO</sup> allele

lacks sequences encoded by exons 2–4, and that it is highly likely that the phenotype observed in homozygote samples results from a loss of Trim33 function, particularly since most of the possible splicings, for example, exon 1 to 4, will lead to a frameshift and premature translational stop. These mutant mRNAs would produce only the very N-terminal peptide encoded by *Trim33* exon 1. Only the splicing from exon 1 to 9 or exon 1 to 13 would maintain the open reading frame. In these unlikely hypothetical cases the protein product would lack the tripartite motif, but would contain the C-terminal PHD and Bromo domains. Based on the current knowledge, it is impossible to say whether these aberrant proteins lacking the functionally important tripartite motif would possess any biological activity.

A recent publication of Dupont *et al.* showed that in *Xenopus* embryos *XTrim33* (*Ectodermin*) plays a critical role in controlling TGF- $\beta$  responses during gastrulation (Dupont *et al.*, 2005). Specifically, these authors demonstrated that *XTrim33* is required for specification of the ectoderm and for restricting the mesoderm-inducing activity of TGF- $\beta$ s. Our finding that *Trim33* null embryos die during somitogenesis demonstrates that *Trim33* is also required for early embryogenesis in mammals. If the role of Trim33 is to negatively regulate Tgf- $\beta$  signaling during embryogenesis, one would expect that *Trim33* null mutants would display phenotypes that are consistent with amplified Tgf- $\beta$  signaling. The observed external *Trim33*<sup>KO/KO</sup> phenotype is very similar to that seen in transgenic mouse embryos that demonstrate ectopic expression of the Tgf- $\beta$  type II receptor (Zwijsen *et al.*, 1999). Another study recently demonstrated that abrogation of *Drap1*, a negative regulator of Nodal, leads to aberrant Nodal signaling and early embryonic lethality (Iratni *et al.*, 2002). Interestingly, similar to *Trim33*<sup>KO/KO</sup> mutants, embryos that are heterozygous for Nodal and lack *Drap1* undergo gastrulation, but die at E9.5. Subsequent studies will show whether the observed phenotype results from defects in ectodermal specification or some other catastrophic mechanistic failure during early embryogenesis. Moreover, mice carrying the floxed *Trim33* allele (*Trim33*<sup>FX</sup>) as described herein can be used to investigate the role of this gene later in development during organogenesis, as well as during postnatal life.

## METHODS

### Generation of Mice Carrying the Floxed and Knockout *Trim33* Alleles

RP2468C16 Bac DNA was used as a template to PCR amplify both short and long arms as *ClaI-XbaI* and *KpnI-SalI* fragments, respectively. High-fidelity Supermix (Qiagen, Valencia, CA) polymerase was used for amplification, and the generated arms were sequenced to verify that no PCR-generated mutations were introduced. A single *loxP* site was inserted into intron 4 by replacing a small 553-bp *SacI-PstI* fragment with a *loxP* sequence

flanked by *SacI* and *PstI* restriction sites. Subsequently, a *loxPNeoloxP* cassette was inserted as a *XbaI-NotI* fragment into the *pKODT* plasmid. A long arm containing the single *loxP* sequence in intron 4 was inserted as a *KpnI-SalI* fragment into *loxPNeoloxP/pKODT*, and finally a short arm was added into the construct as a *ClaI-XbaI* fragment. The targeting vector was electroporated into TVB2 mouse ES cells, and recombinant ES cell clones were selected with *G418* as described (Kaartinen *et al.*, 2004, 1995). Mouse chimeras were generated by injecting correctly targeted ES clones into C57BL/6J mouse blastocysts. The floxed *Trim33* allele (*Trim33*<sup>FX</sup>) and the knockout *Trim33* allele (*Trim33*<sup>KO</sup>) were generated by crossing female mice homozygous for the targeting vector with *Ell1a-Cre* transgenic male mice (Xu *et al.*, 2001), which were obtained from Jackson Laboratories (Bar Harbor, ME).

### PCR Screening, Clone Verification, and Genotyping

ES cell DNAs were first screened for correct targeting by PCR using a forward primer (a') 5' CACGACACAAA-GAACTGTAG 3' and a reverse primer (b') 5' CAAG-CAAAACCAAATTAAGG 3'. Subsequently, the presence of the single *loxP* site in intron 4 was verified by PCR using a forward primer (c) 5' CATTGTGCTT-CACCTCCTCCTCTCG 3' and a reverse primer (d) 5' GGGAGGGAAAATCTGGCTGAA 3'. *Trim33*<sup>FX</sup> mice were genotyped using forward and reverse primers (a) 5' GCACCTTGATGAGATCTTCCTCCTCC 3' and (b) 5' GAC-GACATACTGGACACCGTA 3', respectively, while *Trim33*<sup>KO</sup> mice were genotyped using forward and reverse primers (a) 5' GCACCTTGATGAGATCTTCCTCCTCC 3' and (d) 5' GGAGGGAAAATCTGGCTGAA 3', respectively.

### Timed Matings and Embryos Analyses

Mice were mated during the dark period of the controlled light cycle. Female mice acquiring vaginal plugs were designated as day 0. At the time interval indicated in respective figures (E8 to E9), females were euthanized by CO<sub>2</sub> and embryos were extracted in PBS (Invitrogen) followed by further analyses. All studies and procedures performed on mice were carried out at the Animal Care Facility of the Saban Research Institute and were approved by the CHLA Animal Care and Use Committee (IACUC). The mice were maintained in mixed genetic backgrounds.

### RT-PCR

Total RNA was isolated from E8 embryos using the RNeasy mini kit (Qiagen), and cDNAs were synthesized by the Omniscript reverse transcription kit (Qiagen) according to the manufacturers' protocols. Subsequently, the cDNAs were analyzed by PCR for *Trim33* expression using the following primer pairs.  $\beta$ -actin was used as a quality and loading control.

*Trim33-ex4-S* 5'GAGTCTGTTGGAACATCTGGTCAGCG3'  
*Trim33-ex7-AS* 5'GGCCTGTGATATCATTCTGCTGCTGT3'  
*Trim33-ex1-S* 5'GGTGTGTCAGCAGAGCTTGCA3'  
*Trim33-ex6-AS* 5'GATAAGGGTGAAGATGGCCACT3'  
*Trim33-ex15-S* 5'ACCTCATGCACAGGTCGGCAAGGAT3'  
*Trim33-ex20-AS* 5'GCTCAAACCTCTGGCAAAGGAGTGAAG3'  
*β-actin-S* 5'GTGGGCCGGTCTAGGCACCAA3'  
*β-actin-AS* 5'CGGTTGCCTTAGGGTTCAGG3'

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